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Immobilization of *Clostridium kluyveri* on wheat straw to alleviate ammonia inhibition during chain elongation for *n*-caproate production

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ABSTRACT

Biosynthesis of *n*-caproate from waste streams rich in acetate and ethanol through chain elongation has offered a potentially sustainable way for future production of liquid biofuels. However, most of the waste streams that fit with the purpose (e.g., digestate) are also rich in ammonium which at high concentration may cause toxic effects on the bioconversion process. This study aims to develop a robust, efficient, and cost-effective chain elongation process with high caproate productivity and tolerance to high ammonia concentration, through immobilization of *Clostridium kluyveri* on biomass particles as immobilization material. The threshold ammonia concentration for suspended cells cultivation was 2.1 g/L, while it was higher than 5.0 g/L for the wheat straw immobilized system. The caproate production process was dependent on the selected carriers and was performing in the order of: wheat straw > grass straw > saw dust. The biofilm immobilized on the wheat straw showed good reuse capability for caproate production under high ammonia concentration. Moreover, the lag phase for caproate production was shortened from 72 to 30 h after 8 times reuse. These results proved that caproate production and tolerance of chain elongation to ammonia toxicity could be enhanced via cell immobilization. This study offers insight into future development of efficient and cost-effective chain elongation system for production of caproate and other value-added products.

1. Introduction

Renewable energy technologies have attracted great interest due to the limitation of fossil energy carriers and the climate challenges the world is facing (Vasudevan et al., 2014). In the past decade, both of solid organic biomass wastes (e.g., food waste and agricultural wastes) and liquid organic wastewaters (e.g., starch and glucose rich wastewater) have been considered as energy source for production of chemicals and biofuels (Farmanbordan et al., 2018; Vergine et al., 2015). Liquid biofuels or chemicals offer advantages in comparison of gaseous ones, such as syngas, as they have higher energy densities and are easier to be stored and transported. Among other products, medium chain fatty acids such as caproate, which have lower oxygen/carbon ratio than shorter chain products, are considered to be more appropriate precursors for biofuels (e.g., hexanol) or biochemicals with high energy density (Zhang et al., 2013b). For instance, caproate can be used as a precursor for the biosynthesis of hexyl esters, which can be further converted to hexanol by esterification and hydrogenation reactions. It can also be used for diverse industrial applications such as food additives, medicine, tobacco flavor, rubber, and dyes (Cheon et al., 2014).

Caproate is usually produced from fossil resources in the chemical industry or separated from natural oils (Liu and Jarboe, 2012; Roghair et al., 2018a). It can also be produced biologically by fermentation process called chain-elongation process using biodegradable organic wastes as substrates (Cavalcante et al., 2017). Several microbes including *Clostridium kluyveri*, *C. sp. BS-1*, *Megasphaera elsdenii*, and *Ruminococcaceae bacterium* CPB6, have been reported as microorganisms for *n*-caproate generation (Cavalcante et al., 2017; Zhu et al., 2017). Among them, the spore-forming *C. kluyveri* seems to be one of the most promising strain that can obtain high caproate titer (110.2 mmol/L) and is resistant to harsh environments (Ding et al., 2010; Weimer and Stevenson, 2012).

Based on reverse-oxidation (RBO) cycles, caproate can be produced through chain elongation during anaerobic fermentation during which ethanol or lactic acid is utilized as an electron donor and acetate is used as electron acceptor (Ge et al., 2015; Seedorf et al., 2008; Roghair et al., 2018b). Caproate formation by *C. kluyveri* from ethanol and acetate can be described by three coupled reactions in Eqs. (1)–(3) (Seedorf et al., 2008). Eq. (4) was obtained from combination of Eqs. (2) and (3), which describes caproate production through butyrate formation

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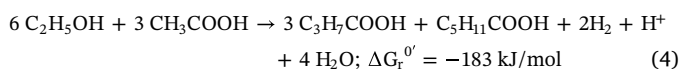
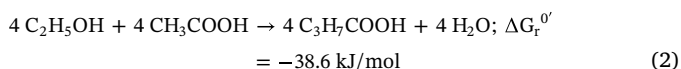
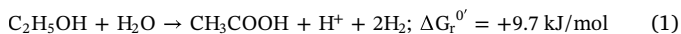
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(Cavalcante et al., 2017).



To improve the competitive ability of caproate biosynthesis in commercial market, utilization of low-cost raw materials is very important. Ethanol and acetate as the main substrates can be generated by anaerobic fermentation/digestion of considerable organic wastes such as food waste, agricultural straw and carbohydrate-rich wastewater (Amiri et al., 2014; Liu and Shonnard, 2014; Zhang et al., 2013a). However, the effluents of anaerobic fermentation/digestion have always a complex composition. They always include acetate, propionate, butyrate, and other inorganic components such as ammonia which at high concentrations can be toxic to microorganisms (Rajagopal et al., 2013). Thus, fermentation for caproate production through chain elongation is apt to be inhibited by ammonia, resulting in low caproate yield and potential process failure. Since many of the potential feedstock for the second generation production of caproate contain high ammonia loads, it is important to elucidate the impact of ammonia on the growth of caproate producing organisms. However, the response of caproate-producing bacteria especially the extensively studied species such as *C. kluyveri* to high ammonia concentration has never been explored. Consequently, it is essential to develop an alternative approach to overcome the ammonia toxicity during chain elongation for caproate production. Cell suspended system is the most common way for conducting chain elongation by *C. kluyveri*. However, suspended cultivation fermentation permits relatively low feed flow rates in continuously fed processes. However, high flow rates would be required to achieve high production rates for caproate. Therefore, immobilization of the microbial cells will facilitate higher flow feeding rates without washing out of the biomass. Furthermore, the suspended cells are more exposed to inhibitory compounds. Comparatively, cell immobilization is a promising technique to enhance the stability and productivity of fermentation. Biofilm could be formed on matrix by adsorption and embedding which is the most common immobilization method. The fixed microorganisms will be able to be retained in the reactor during continuous mode operation even at flow rates higher than the growth rates of the microorganisms, and thereby attaining higher cell concentration for higher productivity (Ma et al., 2017). Selection of an effective carrier for microbial immobilization is essential for the performance of the system. The material should provide good support for microbial growth, have high surface area, be free of toxicants to microbes, and be physically and biologically stable. Other requirements would be the ease of handling, simple immobilization procedures, and cost-effective for being used in large-scale plant (Kumar et al., 2016). Thus, cell immobilization could be adopted to improve the performance of chain elongation for caproate production, which has not been reported. Moreover, the mechanism of biofilm formation on solid carriers by *C. kluyveri* and its response to high ammonia concentration are still unclear.

Thus, in this work, immobilization of *C. kluyveri* on different carriers (i.e., saw dust, wheat straw and grass) that are derived from wastes with broad availability was for the first time explored to improve the process tolerance to ammonia inhibition. Thus the main objectives of this study were to: (i) assess the performance of immobilization system for caproate production catalysed by *C. kluyveri*. (ii) Investigate the influence of high ammonia concentration on caproate production during chain elongation process (iii) evaluate the reuse ability and

stability of the biofilm immobilized on the carrier.

2. Material and methods

2.1. Bacterium cultivation and medium

C. kluyveri (DSM 555) was purchased from the DSMZ (The Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). To ensure high activity of the inoculum, *C. kluyveri* was cultivated for four successive cultivations before being used in batch tests. For the fourth cultivation, *C. kluyveri* which would be used as inoculum was incubated for 6–7 days until the bacteria reached the stationary phase ($\text{OD}: 0.85 \pm 0.03$). The DSM-52 medium was adopted for microbial cultivation. Serum bottles with 100 mL working volume were used for fermentations and 50 mL medium was added in each bottle. Under the environment of mixed gas (80% N_2 and 20% CO_2), the serum bottles with medium (except ethanol, NaHCO_3 , vitamin, L-Cysteine-HCl H_2O and $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$) was boiled for 1 min and cooled to room temperature. Thereafter 1.0 mL ethanol was injected into the serum bottle. Prior to sterilization, all bottles were sealed with rubber stoppers and aluminum caps to insure the anaerobic environment. After sterilization, NaHCO_3 , vitamin, L-Cysteine-HCl- H_2O and $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ solutions which were filtered through sterilized filters with 0.45 μm membrane, were injected into the bottles. The pH value was adjusted to 6.8 ± 0.1 before inoculation. After inoculation (10%), all the bottles were put on a shaker (KS 501, IKA) with 104 rpm, and operated at constant temperature of 37 °C.

Each liter medium contained 10 g CH_3COOK , 0.31 g K_2HPO_4 , 0.23 g KH_2PO_4 , 0.25 g NH_4Cl , 0.2 g $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$, 1.0 g yeast extract, 1.0 mL trace element solution, 1.0 mL selenite-tungstate solution, 0.5 mL Naresazurin solution (0.1% w/v), 20 mL ethanol (99.9%), 2.5 g NaHCO_3 , 1.0 mL vitamin solution, 0.25 g L-Cysteine-HCl H_2O , 0.25 g $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$. The composition (per liter) trace element solution was 10 mL HCl (25%, 7.7 M), 1.50 g $\text{FeCl}_2 \cdot 4 \text{H}_2\text{O}$, 70 mg ZnCl_2 , 100 mg $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 6 mg H_3BO_3 , 190 mg $\text{CoCl}_2 \cdot 6 \text{H}_2\text{O}$, 2 mg $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 24 mg $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 36 mg $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ and 990 mL distilled water. The composition (per liter) of the selenite-tungstate solution was 0.5 g NaOH, 3.0 mg $\text{Na}_2\text{SeO}_3 \cdot 5\text{H}_2\text{O}$, 4.0 mg $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$ and 1000 mL distilled water. Finally the vitamin solution contained 100 mg Vitamin B_{12} , 80 mg *p*-Aminobenzoic acid, 20 mg D(+)-Biotin, 200 mg Nicotinic acid, 100 mg Calcium pantothenate, 300 mg Pyridoxine hydrochloride, 200 mg Thiamine-HCl- $2\text{H}_2\text{O}$ and 1000 mL distilled water.

2.2. Batch tests

2.2.1. Influence of immobilization carrier and ammonia concentration on chain elongation

To investigate the influence of immobilization carrier and ammonia on the performance of chain elongation process, batch tests were carried out in suspended and immobilized systems. In batch tests, wheat straw (approx. 1.0 cm) was applied as carrier with the loading of 40 g/L. The DSM-52 medium was used as cultivation medium (with 84.4 mg/L ammonia). Extra ammonia was added in both of cell suspended and immobilized systems in the form of ammonium for testing the effect of ammonia on microbial growth, and it was added at concentrations of 0.4, 0.8, 1.2, and 1.6 g/L. During the fermentation process, the serum bottles of cell suspended system were placed on a shaker, and those of immobilized system were put on a bench without shaking. The procedure of treatment method before inoculation, the fermentation temperature, and the shaker condition were the same as Section 2.1. Each of the groups was conducted in duplicate.

2.2.2. Influence of immobilized carrier loading on fermentation performance

Since immobilization carrier could provide large surface area for microbial growth, the loading of immobilization carrier would have

distinct influence on the amount of biofilm might be important parameter affecting caproate production. To determine the optimum loading of immobilization carrier, different loadings of wheat straw (approx. 0.5 cm) were designed, being 10, 20, 30, 40, 50 and 60 g/L. The DSM-52 medium was used as cultivation medium and no extra ammonia was added.

2.2.3. Influence of immobilized carrier on caproate production

Three different types of materials including wheat straw, saw dust and grass straw were selected as immobilized carrier. Wheat straw and grass straw were cut into pieces (approx. 1.0 cm) before being used. No further pretreatment was applied out on saw dust since its particle size was below 1.0 cm. All the immobilized carriers were dried at 100 °C until their weight was constant and their loadings were all designed as 40 g/L, as it was the optimum loading determined in Section 3.2.

To determine the optimum immobilization carrier, batch tests were carried out in four different systems including suspended growth, and immobilization on wheat straw, grass straw, or saw dust. For determining the ammonia toxicity on *C. kluyveri* to high ammonia concentrations, the range of 2 to 5 g/L was applied for each system.

2.3. Reuse of immobilized biofilm

The possibility of recycling the immobilized biofilm for caproate production was investigated using two different cultivation media: DSM-52 medium (0.01 g/L ammonium) and DSM-52 medium with 2 g/L extra ammonium addition (total ammonia of 2.1 g/L). The fermentation duration was 6 days for the first batch and 4 days for the following seven batches. For each new batch cycle, the serum bottle was refilled with new medium under the 100% nitrogen gas environment while the carrier which immobilized the biofilm was kept in the bottle for the next batch test. The pH was adjusted to 6.8 ± 0.1 before starting the batch runs. To observe the variation of substrates and products in anaerobic system, the 8th batch was selected and the concentrations of acetate, ethanol, butyrate, caproate, and hydrogen of this batch were detected.

2.4. Analytical methods

The pH values of liquid samples were detected by a pH meter (Meterlab PHM210, HACH, USA). Prior to VFA detection, 1.5 mL liquid sample was mixed with 0.1 mL H_3PO_4 solution (34%, v/v). After mixing, the mixture was centrifuged at 10,000 rpm for 10 min. VFA and alcohols were detected by a gas-chromatograph (GC-HP5890, Thermo) equipped with a FFAP fused silica capillary column (30 m \times 0.53 mm, film thickness 1.5 mm) and a flame ionization detector (FID). The carrier gas was N_2 . Hydrogen was analyzed by a GC (GC 1300, Thermo) equipped with TCD and fitted with a 4.5 m \times 3 mm stainless column packed with Molsieve SA (10/80). The temperatures of the injector, detector and oven were 190 °C, 130 °C, and 190 °C, respectively. N_2 was used as carrier gas.

For visualization of *C. kluyveri* with scanning electron microscope (SEM), cells were prepared by fixing with 4% formaldehyde overnight in 4 °C and dehydrating with successive passage through gradient 25%, 50%, 75%, 95% and 100% ethanol and freeze dried subsequently. The prepared samples were coated by nano-gold (Quorum sputter coater, UK) and observed with the SEM (Quanta 200 ESEM FEG, FEI, USA).

3. Results and discussion

3.1. Influence of ammonia on caproate production in cell suspended and immobilized systems

The influence of low ammonia concentration on caproate production is shown in Fig. 1A and B. Lag phase of 104 h was observed in suspended system without ammonia addition, which was shortened to

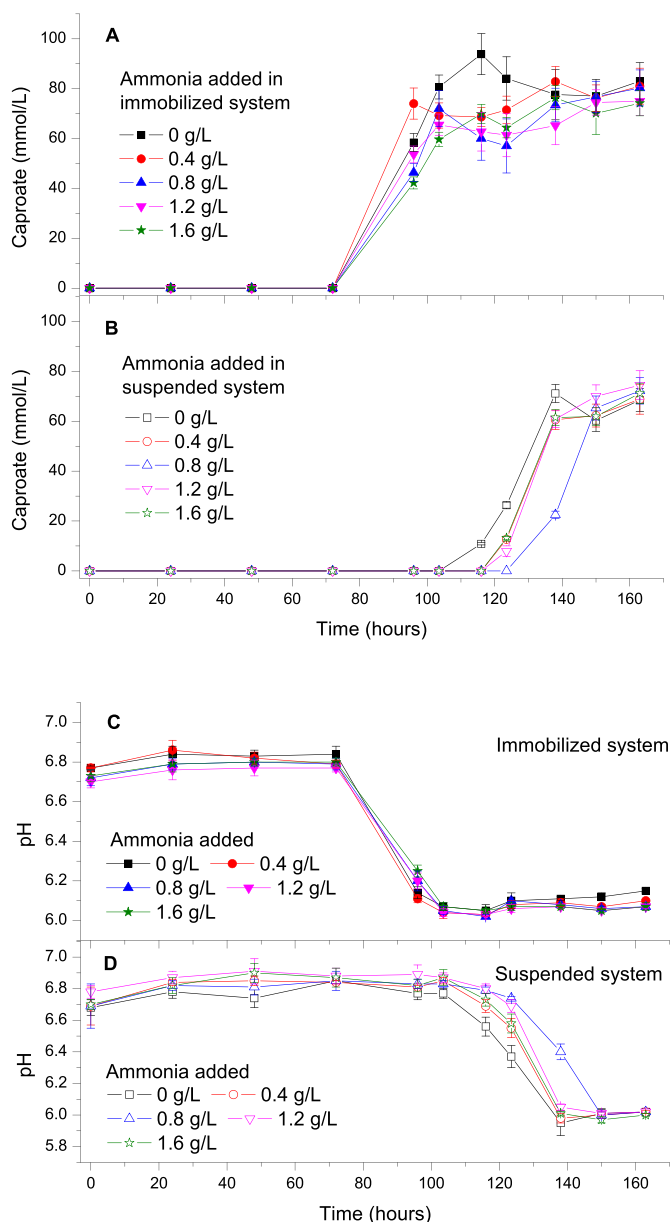


Fig. 1. Profile of caproate production and pH, during chain elongation process by *C. kluyveri*. Wheat straw (< 1.0 cm) was applied as carrier with the loading of 40 g/L.

72 h when wheat straw was added in the fermenter. Addition of ammonia in medium resulted in a much longer lag phase, i.e., the lag phase was 116 h when 0.4 g/L ammonia was added in cell suspended system. However, with same ammonia concentration shorter lag phase was observed in immobilized system, corresponding to 72 h. The results indicated that addition of immobilized carrier could shorten the lag phase. Noted is that the caproate concentration will decrease after it achieve the maximum value. It is therefore needed to determine the harvest time to obtain the maximum caproate production. The harvest time and the corresponding caproate production are shown in Table S1. Compared with cell suspended system, the caproate production was enhanced in immobilized system. For instance, without addition of extra ammonia, the maximum caproate was 71.2 mmol/L in cell suspended system, while it was 93.8 mmol/L in immobilized system, corresponding to 31.7% improvement. These results illustrated that addition of immobilized carrier in fermenter could shorten the lag phase and improve caproate production, and could counteract ammonia

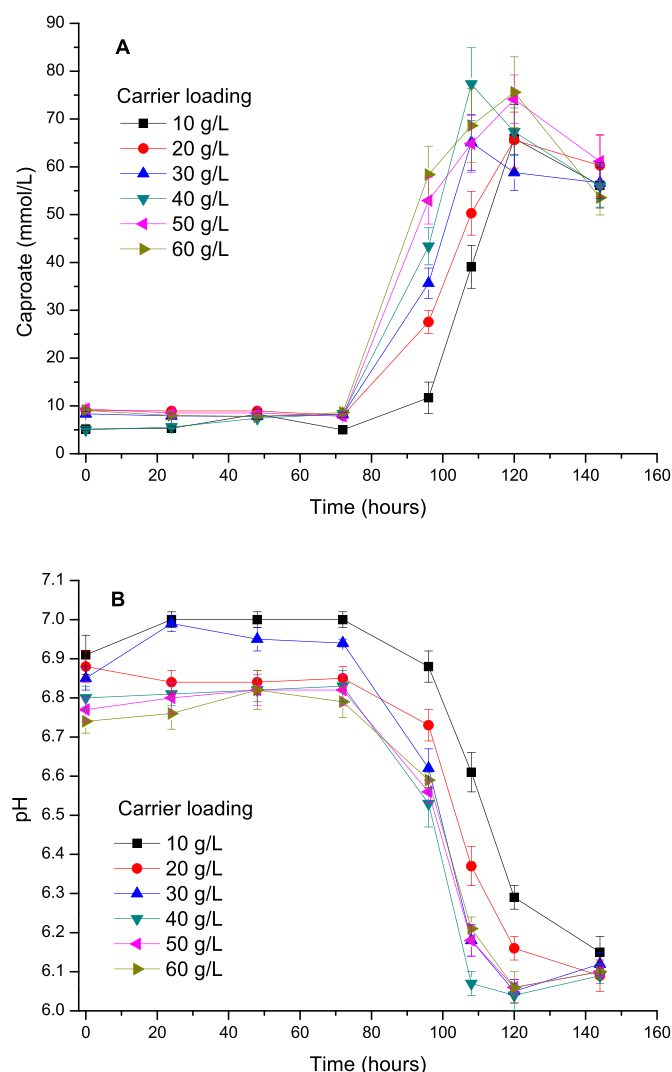


Fig. 2. Influence of immobilization carrier loading on caproate production (A: cumulative caproate; B: pH). No extra ammonia was added in addition to DSM-52 medium.

inhibition.

Variation of pH during anaerobic fermentation is shown in Fig. 1C and D. The initial pH was in the range of 6.68–6.78. During lag phase, the pH value maintained at a stable level. However, sharp decrease in pH was observed after lag phase. After 7 days of fermentation, the pH values decreased to stable levels around 6.0 for suspended system and 6.1 for immobilized system, respectively. The sharp decrease of pH might be due to acids formation as indicated by Eq. (4). Three moles of acid (acetate) were converted and 4 mol acids (butyrate and caproate) were generated. Meanwhile, 1 mol H^+ was produced, meaning more free acids would exist in fermenter. Therefore, pH could also be used as an indicator to determine whether reaction (1) happened or not.

3.2. Influence of carrier loading on caproate production

The immobilized carrier could provide a large area for microbial growth and propagation. However, excess amount of carrier might affect the mass transfer in the system and therefore result in the lower fermentation performance. To optimize the loading of immobilized carrier, a batch test was conducted. The influence of immobilization carrier loading on caproate production is shown in Fig. 2 and Table S2. From Fig. 2A, the lag phase with the carrier loading of 10 g/L was longer than the other groups during caproate production,

demonstrating that increasing carrier concentration was in favor of shortening lag phase. However, there was no remarkable difference in lag phase duration (72 h), when the carrier loading was in the range of 20 to 60 g/L. Moreover, the influence of immobilization carrier loading on caproate production was observed. From Table S2, the maximum caproate (77.3 mmol/L) was obtained at the carrier loading of 40 g/L after 108 h fermentation. As shown in Fig. 2B, the pH increased from 6.9 to 7.0 at the initial period of fermentation when the carrier loading was increased from 10 to 20 g/L. In contrast, the pH in the reactors with higher carrier loadings was maintained at stable level. The above results showed that addition of higher amount of carrier was in favor of system stability. In addition, the pH decreased with the generation of caproate. The lowest pH of 6.04 was observed in the reactor with carrier loading of 40 g/L at 120 h.

Theoretically, more biofilm would be formed when more amounts of immobilization carrier were added. Ethanol and acetate then would be converted to caproate more efficiently with more biofilm. However, as a result, more substrates would also be needed for microbial cell growth, resulting in decreasing of substrates availability as well as the caproate production. Moreover, the more immobilization carrier in the system, the lower mass transfer efficiency would be. Overload of carrier might negatively affect the mass transfer efficiency of substrates and nutrients to the cells, which will limit the rate of caproate production. On the other hand, the cells on the biofilm may also be protected, avoiding the sharp decrease of pH during caproate production. In Fig. 2A and Table S2, the maximum caproate concentrations were observed at 120 h at the loadings of 50 and 60 g/L, while the same level of production were achieved at 108 h at the loadings of 30 and 40 g/L. The maximum caproate concentration of 77.3 mmol/L (8.98 g/L) was obtained at the loading rate of 40 g/L, illustrating that the optimum loading for wheat straw was 40 g/L. It was worth to mention that the maximum production of 77.3 mmol/L obtained from small particle size (approx. 0.5 cm) was lower than the data (93.8 mmol/L) obtained from big particle size (approx. 1.0 cm). This finding suggested wheat straw with particle size around 1.0 cm performed better productivity in immobilization system. This trend could be explained as a combination of the positive effects of immobilization (i.e., protection of the cells on the biofilm) and the negative impact of deteriorated mass transfer.

3.3. Response of immobilized system with different carriers to high concentration of ammonia

To investigate the influence of different carriers on caproate production and how the carriers influence ammonia inhibition, wheat straw, grass straw and saw dust were selected as carriers for immobilization. The media for the fermentation were amended with ammonia additions in the range of 2–5 g/L addition. The results obtained from suspended and immobilized systems with different ammonia concentrations are shown in Table 1. After 195 h of fermentation at ammonia concentration of 2.0 g/L, no caproate was detected in liquid samples in both of suspended and saw dust based immobilization systems. Thus, it was concluded that addition of 2.0 g/L ammonia to

Table 1

Caproate production from suspended and immobilized systems with varied ammonia concentration (data \pm standard deviation).

Test no.	Ammonia addition (g/L)	Caproate production (mmol/L)			
		Suspended system	Immobilized carriers		
			Wheat straw	Grass straw	Saw dust
1	2	3.7 \pm 0.1	64.7 \pm 6.7	59.5 \pm 4.3	3.5 \pm 0.2
2	3	4.0 \pm 0.2	63.5 \pm 5.1	49.6 \pm 2.8	3.4 \pm 0.1
3	4	3.9 \pm 0.2	53.8 \pm 4.0	39.8 \pm 1.8	3.9 \pm 0.3
4	5	3.4 \pm 0.3	12.7 \pm 1.7	3.6 \pm 0.9	3.6 \pm 0.2

suspended system inhibited the activity of *C. kluyveri* completely, and the corresponding threshold ammonia concentration was 2.1 g/L. Compared with saw dust, wheat straw and grass straw based systems showed a better tolerance to high ammonia concentration. But significant difference on caproate concentration can also be observed (Student-*t*-test, $p < 0.02$). The maximum caproate concentration of 64.7 mmol/L was obtained when wheat straw was used as immobilized carrier with 2.0 g/L ammonia addition, indicating that wheat straw was more efficient carrier to improve the tolerance of *C. kluyveri* to a severe environment with high ammonia concentration.

The pH could also be used as an indicator for caproate production. As shown in Fig. S1, the pH of suspended and saw dust based immobilization systems maintained at stable levels (i.e., 7.0 ± 0.1 for suspended system and 6.8 ± 0.1 for saw dust immobilization system), because the microbes in these two systems were completely inhibited. In the wheat straw and grass straw based immobilization systems, the pH decreased in the systems when the cells started to generate caproate. In wheat straw based immobilization system, it was noted that the lag phase extended at higher ammonia concentration in comparison of that at lower ammonia concentration (Fig. 1). For example, the lag phase prolonged from 72 to 92 h when ammonia concentration increased from 1.6 to 2.0 g/L.

Fig. 3A and B shows the surface structure of wheat straw covered by *C. kluyveri* cells. The cell length of *C. kluyveri* was in the range of 5–6 μm when 2 g/L ammonia was added (Fig. 3A). Results also showed microbes grew on both of the crude and glossy surface of wheat straw and formed biofilm. In addition, wheat straw could provide a large surface

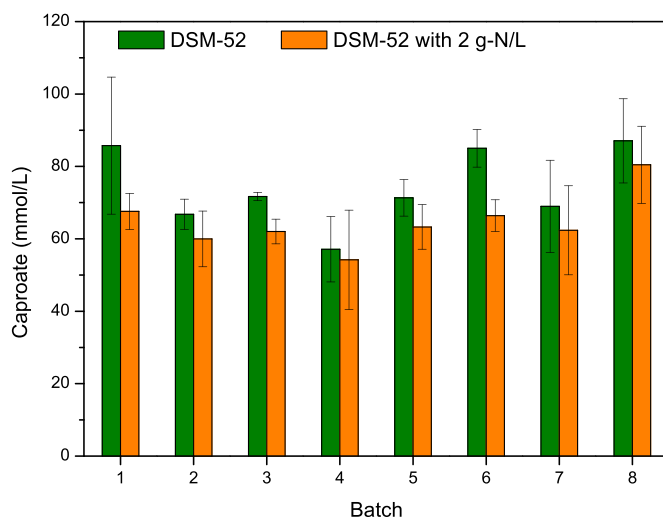


Fig. 4. Caproate production from different batches using the wheat straw covered by biofilm.

for microbe growth not only outside of profile but also inside of the porous structure. The diameter of the porous structure which was observed on the cross section of wheat straw varied from 3.5 to 22 μm (Fig. 3C). The considerable holes could enhance the surface area of wheat straw. Microbes can grow both in outside and inside of these

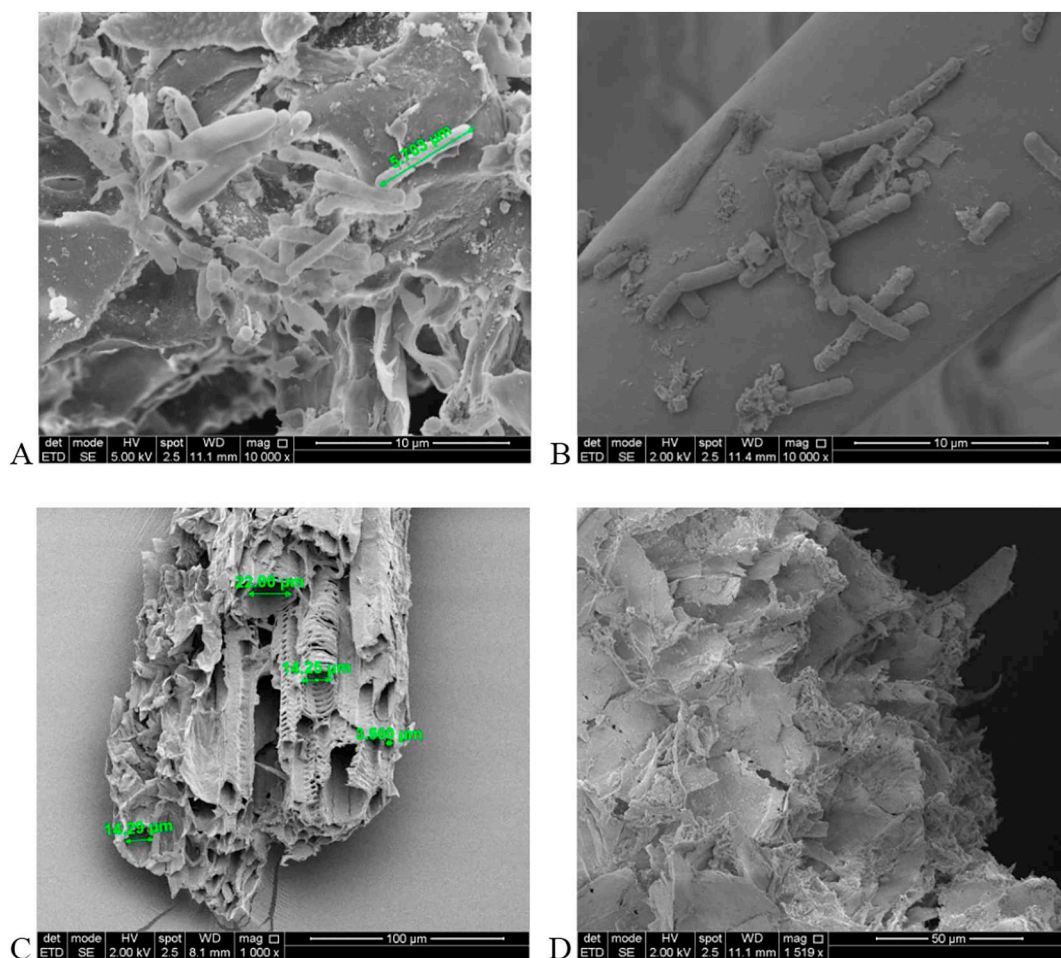


Fig. 3. Surface structure of wheat straw covered by *C. kluyveri* (A: immobilized cells on the crude surface of wheat straw with 2 g/L ammonia addition; B: immobilized cells on the glossy surface of wheat straw with 2 g/L ammonia addition; C: cross section of wheat straw soaked in distilled water for 7 days; D: biofilm formed on the cross section of wheat straw with 2 g/L ammonia addition).

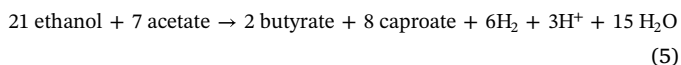
holes, forming a stable biofilm on the surface of wheat straw (Fig. 3D). These results showed that wheat straw could be an ideal immobilization carrier for biofilm formation.

3.4. Biofilm recycling for caproate production

As proved in the previous section, biofilm formed when immobilization carrier was added into fermentation system. To obtain high caproate production, the stability and activity of biofilm are of great importance. Although biofilm was clearly observed on wheat straw, it was unclear whether the biofilm could be reused for a subsequent cultivation. Fig. 4 shows the caproate production when the biofilm was reused for eight batches. It was observed that the caproate production in the first batch were 85.7 and 67.6 mmol/L for the system without and with 2 g/L ammonia addition, respectively. A slight decrease in caproate production was observed when the biofilm was reused for seven times. In the eighth batch, caproate productions of the DSM-52 medium without and with 2 g/L ammonia addition were 86.9 and 80.1 mmol/L, respectively. These results proved that the biofilm formed on wheat straw performed high stability and activity for converting acetate and ethanol to caproate. Moreover, the results shown in Fig. 4 also indicated that the caproate production without ammonia addition in the medium was significantly higher than that with 2 g/L ammonia (Student-*t*-test, $p < 0.02$), which demonstrates that ammonia inhibits the chain elongation process. The average caproate productions were 74.2 mmol/L for medium DSM-52 and 64.6 mmol/L for medium DSM-52 with 2 g/L ammonia addition, respectively. Although the average production of caproate decreased 13% when 2 g/L ammonia was added into DSM-52 medium, the biofilm provided higher tolerance to ammonia inhibition compared to the cell suspended system.

Fig. 5 shows the change of ethanol, acetate, butyrate, caproate and hydrogen in concentrations during the time course of a fermentation process. Lag phase was obviously shortened when biofilm was reused. From Fig. 5A, the lag phase was 15 h in the immobilized system which was shortened by 5.3-fold in comparison of cell suspended system (104 h as shown in Fig. 1). In contrast, the lag phase was 30 h for the medium with 2 g/L ammonia addition, which further proved that higher ammonia is negatively affecting fermentation, increasing the lag phase duration. However, the lag phase was obviously shorter than that of the first batch (92 h as shown in Fig. S1). These supports that the biofilm formed on wheat straw result in higher productivity for conversion of acetate and ethanol to caproate.

Along with the production of caproate, hydrogen was simultaneously generated during the chain elongation process, as shown in Fig. 5C. The production rate of hydrogen without ammonia addition was higher than that with 2 g/L with ammonia addition. This observation could be explained by the longer lag phase when ammonia was added. Nevertheless no obvious difference on the final hydrogen production was observed. According to previous reports (Seedorf et al., 2008; Venkateswar et al., 2017), hydrogen was produced when ethanol was converted into acetate, as described in Eq. (1). The fluxes of Eq. (1) were 32.5 and 31.6 mmol/L for the two systems with different media, as shown in Table 2. Thus the total acetate which was used to generate butyrate comprised of the amount that was converted from ethanol and the amount added in medium. It can be calculated that the fluxes of Eq. (2) for the two systems were 98.8 and 97.9 mmol/L, respectively. The generated butyrate were further used for caproate production via Eq. (3), corresponding to the fluxes of 78.9 and 78.2 mmol/L, respectively. According to the fluxes of Eqs. (1)–(3), the general reaction for caproate production from acetate and ethanol can be described according to Eq. (5).



The carbon conversion ratio was 85.7%, which was improved by 157.3% compared to Eq. (4). Caproate generated through a carboxylic

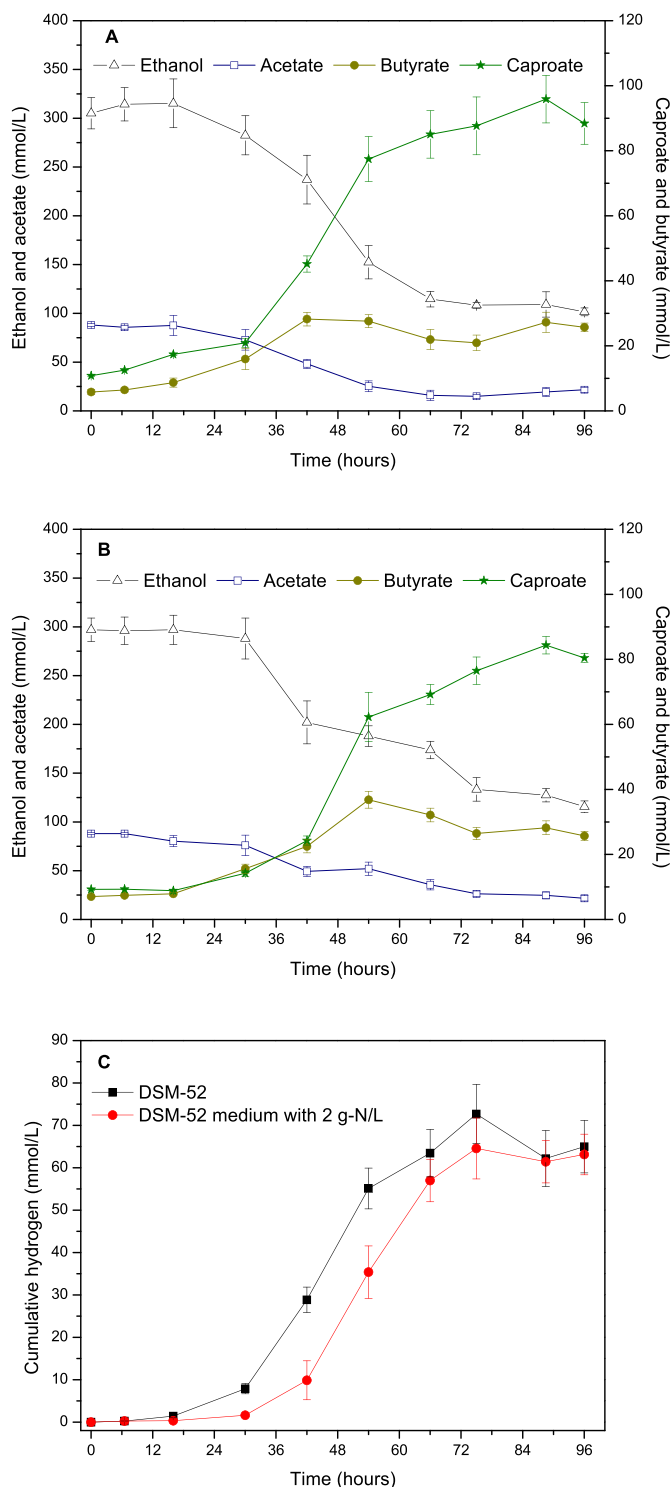


Fig. 5. Profile of substrate and product concentrations during the eighth batch cultivation (A: DSM-52 medium; B: DSM-52 medium with 2 g/L ammonia addition; C: cumulative hydrogen).

acid chain elongation process, which uses reverse β -oxidation of acetate and/or butyrate, and ethanol or lactate as an electron donor (Kucek et al., 2016; Zhang et al., 2013b). In this study, butyrate accumulated along with generation of caproate. As a carbon source, butyrate was also converted into caproate by *C. kluyveri* using ethanol as electron donor. Butyrate utilization might be the main reason for higher caproate production and low concentration of butyrate in our research.

Caproate production from chain elongation process can be

Table 2
Substrate consumption and products generation in the immobilized system.

Item	Unit	DSM-52 medium	DSM-52 medium with 2 g/L ammonia addition
Consumption of ethanol	mmol/L	203.6	181.5
Consumption of acetate	mmol/L	66.3	66.3
Butyrate production	mmol/L	19.9	19.7
Caproate production	mmol/L	77.6	71.1
H ₂ production	mmol/L	65.0	63.1
Flux of Eq. (1) ^a	mmol/L	32.5	31.6
Flux of Eq. (2) ^a	mmol/L	98.8	97.9
Flux of Eq. (3) ^a	mmol/L	78.9	78.2

^a Practical flux of the reaction.

influenced by several parameters, including pH, concentration of substrates, inoculum activity, operation mode, and even configuration of reactor. Among of these factors, the concentration of ethanol which is used as electron donor is one of the key parameters influencing caproate production. A study conducted by Yin et al. (2017) found that the caproate production could be enhanced by increasing ethanol concentration, and the caproate production of 72.5 mmol/L was achieved with an ethanol/acetate ratio of 10:1 (550 mmol/L total carbon) in treating high ethanol strength wastewater. A much higher caproate production reported previously was 110.2 mmol/L with addition of 700 mmol/L ethanol and 120 mmol/L acetate (Weimer and Stevenson, 2012). In fact, it is hard to achieve the caproate to a production exceeding 103 mmol/L even though the pH was controlled at 7.0. Steinbusch et al. (2011) obtained the highest caproate production up to 71.2 mmol/L using ethanol as electron donor (pH 7.0). In the work of Grootcholten et al. (2013), continuous operation (9.0 g/L/d acetate and 20.7 g/L/d ethanol) was carried out in an upflow anaerobic filter and the highest caproate concentration of 103.3 mmol/L (12 g/L) was obtained after fermentation of 32–36 days, with the pH being controlled in the range of 6.5–7.2. In addition, the type of electron donor will also influence the production of caproate. In a system which lactate was used as electron donor, the caproate production achieved as high as 201.4 mmol/L, with inoculum of fermentation pit from Chinese strong flavor liquor production (Zhu et al., 2015). Although caproate production could be improved by optimizing operation condition, the suspended cells are easy to be washout if operation was conducted in continuous mode. Also, suspended cells are susceptible to adverse environment, e.g., inhibition by ethanol would occur at concentrations exceeding 700 mmol/L (Yin et al., 2017). To improve strengthen tolerance of microbial cells to toxicity, Liu et al. (2017) provided biochar as matrix for microbial growth in a mixed culture system in which *C. kluyveri* dominated the microbial community, achieving a much higher caproate production of 181.6 mmol/L. The higher amounts of extracellular polymer substances and higher conductivity induced by biochar explained the reinforcement effect in chain elongation process. Though these studies reported on the same strain and product, none of them have ever investigated the ammonia toxic and explored the strategy to alleviate such inhibition. In this study, wheat straw was applied as matrix for microbes to convert acetate and ethanol to caproate, with the purpose to alleviate the inhibition caused by ammonia. Our work bring the understanding of caporate biosynthesis in next level and offer an efficient and cost-effective way to alleviate the ammonia inhibition by cell immobilization reusing waste materials (i.e., wheat straw). And also, biofilm formed on wheat straw could be recovered for batches and was potentially utilized in continuous anaerobic fermentation. Since caproate production could be influenced by various parameters, the objective of this study was not to reach the highest production, but in the future it is definitely interesting to investigate how the immobilization affects the caproate production in other systems.

Inorganic ammonia nitrogen in aqueous solution exists in two

forms: ammonium ion and free ammonia. It was reported that free ammonia is the main inhibitor because it diffuses into the bacteria cells freely which can cause proton imbalance, increase maintenance energy requirements, change intracellular pH and inhibit specific enzyme reactions (Wang et al., 2016). The free ammonia concentration can be affected by temperature and pH, e.g., it increase with an increase in pH (Cecilia et al., 2018). In the immobilization system, the mass transfer from liquid to cells will decrease. Thus the cells on the biofilm can be protected when the ammonia centration is high. However, there was no protection for cells in suspended system. The suspended cell was prone to be inhibited by ammonia. The protection of cells by wheat straw might be one of the reasons for high activity of immobilized cells.

Wheat straw is a preferred matrix for cell immobilization due to its advantages: easily available, cost effective, free of toxic to microbes, sustaining recovery and biofilm stability. Several biofilm formation mechanisms were reported previously, in which adsorption and embedding are mostly used (Ma et al., 2017; Singh and Singh, 2013; Zhang et al., 2017). In this research, SEM results showed that the porous structure of wheat straw and the large specific surface area were in favor of not only adsorption but also embedding for microbes. It was concluded that both adsorption and embedding contributed to the biofilm formation. Compared to free cells, biofilm was more stable and in high activity, and could perform high tolerance ability to adverse environment. With biofilm growing on the surface of carrier, the intercellular transmission between cells to cells might be strengthened because of the aggregative growth of cells. The butyrate generated from chain elongation process in one cell could be transformed to another cell where it can be directly utilized to produce caproate with ethanol. As a result, the metabolic pathway of *C. kluyveri* may have been changed. This might be the reason for the low butyrate concentration in the effluent. Our research provided a platform for *C. kluyveri* to use complex substrates such as digestate which not only contain ethanol and acetate but also inhibitor to microbes. The digestate will be used as substrate for the caporate production. The effect of mix culture in the digestate on the chain elongation process should be further studied.

4. Conclusions

Wheat straw provided a good immobilization carrier for *C. kluyveri*. The biofilm formed demonstrated high activity for caproate production and provided tolerance to ammonia inhibition. The structure of wheat straw was not disrupted by microbial activity and thus, it could be used as a stable matrix for microbial growth. Compared with suspended cells system, the lag phase was shortened by 5.3-fold. The average caproate productions were 74.2 mmol/L for DSM-52 medium and 64.6 mmol/L for DSM-52 medium with 2 g/L ammonia addition, respectively. Butyrate utilization, the porous structure and large specific surface area of wheat straw contributed to the higher performance of chain elongation and higher caproate production.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envint.2019.03.032>.

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